Different expression of leptin and IGF1 in the adult and prepubertal testis in dogs

Müller, Linda ; Kowalewski, Mariusz P ; Reichler, Iris M ; Kollár, Eszter ; Balogh, Orsolya

Abstract: Leptin (Lep) and insulin-like growth factor 1 (IGF1) are implicated in the regulation of testicular function, but in dogs, our knowledge is limited to the possible role of the IGF1 system in testicular tumors. In this study, we aimed to describe and compare gene expression and protein localization of Lep, IGF1 and their receptors (LepR and IGF1R, respectively) in the testis of healthy adult and prepubertal dogs. Testes were collected from sexually healthy mature (n=7) and from 8-week-old dogs (n=7). Relative gene expression of Lep, LepR, IGF1 and IGF1R was determined by semi-quantitative real-time (TaqMan) PCR and cellular distribution in the testis by immunohistochemistry. Statistical analysis was carried out with Student's t-test. Lep and LepR mRNA concentration was similar between the two groups, but IGF1 and IGF1R gene expression was significantly higher in the 8-week-old pups. Protein localization and the intensity of signals differed by age. In adults, Lep and LepR immunoreactivity was detected in spermatocytes and spermatids. Leydig cells showed sporadic, weak Lep staining. In prepubertal animals, intense Lep signals were present in Leydig and Sertoli cells, and LepR was found in Leydig cells. IGF1 and IGF1R protein was expressed in spermatogonia of the mature testis; IGF1 signals in Leydig cells seemed stronger than IGF1R. In the pups, IGF1 and IGF1R staining was detected in Leydig cells and in gonocytes. Sertoli cells showed weak IGF1 and sporadic, weak IGF1R signals. In conclusion, Lep and IGF1 may support spermatogenesis in adult dogs and mediate Leydig cell function. In the immature testis, they may promote development of Sertoli and Leydig cells and gonocytes.

DOI: https://doi.org/10.1111/rda.12896

Originally published at:
Müller, Linda; Kowalewski, Mariusz P; Reichler, Iris M; Kollár, Eszter; Balogh, Orsolya (2017). Different expression of leptin and IGF1 in the adult and prepubertal testis in dogs. Reproduction in Domestic Animals, 52(Suppl 2):187-192.
DOI: https://doi.org/10.1111/rda.12896

Different expression of leptin and IGF1 in the adult and prepubertal testis in dogs

Linda Müller[a], Mariusz P Kowalewski[b], Iris M Reichler[c], Eszter Kollár[a], Orsolya Balogh[c§]

[a]Department and Clinic of Reproduction, University of Veterinary Medicine, Budapest, Hungary;

[b]Institute of Veterinary Anatomy, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland;

[c]Clinic of Reproductive Medicine, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland

[§]Corresponding author

obalogh@vetclinics.uzh.ch

25

26 **Contents**

27 Leptin (Lep) and insulin-like growth factor 1 (IGF1) are implicated in the regulation of
28 testicular function, but in dogs, our knowledge is limited to the possible role of the IGF1
29 system in testicular tumors. In this study, we aimed to describe and compare gene expression
30 and protein localization of Lep, IGF1 and their receptors (LepR and IGF1R, respectively) in
31 the testis of healthy adult and prepubertal dogs. Testes were collected from sexually healthy
32 mature (n=7) and from 8-week-old dogs (n=7). Relative gene expression of Lep, LepR, IGF1
33 and IGF1R was determined by semi-quantitative real-time (TaqMan) PCR and cellular
34 distribution in the testis by immunohistochemistry. Statistical analysis was carried out with
35 Student's t-test. Lep and LepR mRNA concentration was similar between the two groups, but
36 IGF1 and IGF1R gene expression was significantly higher in the 8-week-old pups. Protein
37 localization and the intensity of signals differed by age. In adults, Lep and LepR
38 immunoreactivity was detected in spermatocytes and spermatids. Leydig cells showed
39 sporadic, weak Lep staining. In prepubertal animals, intense Lep signals were present in
40 Leydig and Sertoli cells, and LepR was found in Leydig cells. IGF1 and IGF1R protein was
41 expressed in spermatogonia of the mature testis; IGF1 signals in Leydig cells seemed stronger
42 than IGF1R. In the pups, IGF1 and IGF1R staining was detected in Leydig cells and in
43 gonocytes. Sertoli cells showed weak IGF1 and sporadic, weak IGF1R signals. In conclusion,
44 Lep and IGF1 may support spermatogenesis in adult dogs and mediate Leydig cell function. In
45 the immature testis, they may promote development of Sertoli and Leydig cells and gonocytes.

46

47

48

Introduction

Leptin (Lep), insulin-like growth factor 1 (IGF1) and their respective receptors (LepR, IGF1R) are present in the testis, which implies that both hormones exert direct actions on the male gonad. There are differences among species in cellular localization of these proteins and thus in function. Furthermore, the distribution pattern of Lep, IGF1 and their receptors also varies according to the developmental stage of the testis. In mice, Lep and LepR were found in gonocytes and spermatogonia during early postnatal life, and the localization changed to spermatocytes from postnatal day 20 on through adulthood (El-Hefnawy et al., 2000; Herrid et al., 2008a). Lep was not detected in interstitial cells of adult mice (Herrid et al., 2008a), while LepR had weak or no signals in interstitial cells (El-Hefnawy et al., 2000). In adult rats, LepR mRNA was localized in Leydig and Sertoli cells and possibly in germ cells (Tena-Sempere et al., 2001). In contrast to mice, Lep and LepR were expressed in Leydig cells both in 2-3 months old and adult pigs, however cells within the seminiferous tubules of the immature testis were negative, while intense signals for both proteins were detected in elongating spermatids in adult animals (Rago et al., 2009).

IGF1 was detected in spermatogenic and Sertoli cells with similar intensity in the immature rat testis, but Sertoli cells became non-reactive by 3 weeks of age. In adult rats, IGF1 was present in spermatocytes, while spermatogonia, Sertoli and Leydig cells were negative (Hansson et al., 1989). Phosphorylated IGF1R however was present in germ cells as well as in Leydig and Sertoli cells (Colón et al., 2007). In horses, the intensity of IGF1 and IGF1R expression in Leydig cells, spermatogonia and spermatocytes was changing from the prepubertal to the adult stage (Yoon et al., 2011). In mature stallions, Leydig cells and spermatogonia showed positive

72 immunoreactivity for both proteins, spermatocytes had only faint signals, and Sertoli cells
73 were negative (Yoon et al., 2011).
74
75 Testicular steroidogenesis as well as germ cell and Sertoli cell function may be modulated by
76 Lep and IGF1 either as autocrine/paracrine or endocrine signals. Tena-Sempere and colleagues
77 (1999 and 2001) showed that *in vitro*, Lep dose- and time-dependently decreased testosterone
78 (T) output from adult rat testicular tissue by down-regulating the hCG-stimulated mRNA
79 expression of several genes in the steroidogenic pathway e.g. steroidogenic acute regulatory
80 protein (StAR). In contrast to adults, Lep had no effect on *in vitro* T production in prepubertal
81 rats (Tena-Sempere et al., 1999). In mice however, Lep inhibited basal T output from cultured
82 testicular slices in immature animals but not in adults (Herrid et al., 2008b). Lep's role in germ
83 cell development was shown in the leptin-deficient ob/ob mice, in which spermatogenesis was
84 impaired, apoptosis was evidenced especially in pachytene spermatocytes, and fewer Leydig
85 cells were present in the interstitium (Bhat et al., 2006). Lep may also be involved in the
86 proliferation and renewal of type A spermatogonia and in the differentiation and maturation of
87 spermatocytes to spermatids in the murine testis (El-Hefnawy et al., 2000).
88 IGF1 is stimulatory on Leydig cell steroidogenesis and its effects are mediated by up-
89 regulation of StAR expression (Manna et al., 2006; Colón et al., 2007). These actions however
90 are also developmental stage dependent. IGF1 alone did not influence the *in vitro*
91 steroidogenic potential of equine Leydig cells collected either from pubertal or post-pubertal
92 animals, but it increased T synthesis synergistically with LH and in a dose-dependent manner
93 in post-pubertal stallions (Yoon and Roser, 2011). The effects of IGF1 on germ cell function
94 were positive, as it reduced the rate of apoptotic cells and promoted DNA synthesis of
95 spermatogonia in rats (Söder et al., 1992; Ozkurkcugil et al., 2004). IGF1 signaling was shown

to be crucial for Sertoli cell proliferation during late fetal and early neonatal life in mice (Pitetti et al., 2013), and it also stimulated differentiation and maturation of murine Leydig cells (Wang et al., 2003). A dose-dependent increase in the proliferation of immature Leydig cells and a decreased number of apoptotic Leydig cells from immature as well as from adult rats were seen *in vitro* after IGF1 treatment (Colón et al., 2007).

In dogs, Lep's role in testis function has not been investigated, while the expression of members of the IGF system by RT-PCR has been studied in testicular tumors in comparison with the normal testis (Peters et al., 2003). Peters et al. (2003) found that Sertoli cell tumors and seminomas had lower gene expression of IGF1 and IGF binding protein 1 than normal testes. Leydig cell and mixed tumors showed higher IGF1R and IGF binding protein 4 mRNA levels. The authors' conclusion was that as the changes in gene expression in the different tumors were not so dramatic compared to the normal tissue, the IGF system is less likely to have a major role in the maintenance and growth of these tumor types. In the normal canine testis however Lep and IGF1 may be involved in the regulation of spermatogenesis and steroidogenesis. Our goal therefore was to study the mRNA expression and protein localization of Lep, IGF1 and their receptors in the testis of healthy, sexually mature dogs and to compare it to the juvenile testis to investigate possible developmental differences.

Material and methods:

Animals, tissue collections

Testes were collected during routine neutering from healthy, sexually mature dogs without testicular pathology (n=7, age 2-5.5 years, five mixed breeds and two English greyhounds, body weight 12-26 kg) and from prepubertal dogs (n=7, 8-week-old Australian Cobberdogs,

body weight 4.1-6.6 kg). The puppies came from 2 different litters (3 and 4 dogs from each). Tissues were kept in RNAlater® at +4°C (Ambion Biotechnologie GmbH, Wiesbaden) and then stored at -80°C for RNA preservation. For IHC, testes were fixed for 24 h in 10% neutral phosphate-buffered formalin at +4°C, washed daily in phosphate buffered saline for one week, dehydrated in graded ethanol series and embedded in paraffin (Kowalewski et al., 2010).

Gene expression of Lep, LepR, IGF1 and IGF1R in the testis

Total RNA was isolated with TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA), and total RNA concentration was measured with NanoDrop 2000 u.v.–Vis Spectrophotometer (Thermo Scientific,Wilmington, DE, USA). DNAse treatment (RQ1 RNase-free DNase; Promega, Dübendorf, Switzerland) of 200 ng RNA per sample was followed by reverse transcription using reagents from Applied Biosystems (Foster City, CA, USA) according to our protocol (Kowalewski et al., 2006a). Semi-quantitative real-time (TaqMan) PCR for the detection of canine Lep, LepR, IGF1 and IGF1R was carried out using the same primers and TaqMan probes as previously reported (Balogh et al., 2012 and 2015; Kautz et al., 2014), *i.e.*,: Lep (forward): 5′-GGG TCG CTG GTC TGG ACT T-3′, Lep (reverse): 5′-CTG TTG GTA GAT GGC CAA CGT-3′, Lep TaqMan probe: 5′-TCC TGG GCT CCA ACC AGT CCT GAG T-3′; LepR (forward): 5′-CAT TTG CGG AGG GAT GGT T-3′, LepR (reverse): 5′-AGC GGT TTC ACC ACG GAA T-3′, LepR TaqMan probe: 5′-TTG ACT CTT CAC CAA CGT GTG TGG TTC C-3′; IGF1R (forward): 5′-GGA CGT TGA GCC TGG CAT T-3′, IGF1R (reverse): 5′-CAC TCT TAG CCC CAC GGA TGT-3′, IGF1R TaqMan probe: 5′-AGC CCT GGA CGC AGT ATG CGG-3′. TaqMan probes were labeled at the 5'-end with the reporter dye 6-carboxyfluorescein (FAM), and at the 3'-end with the quencher 6-carboxytetramethyl-rhodamine (TAMRA). Efficiency of the PCR reactions, which was established with the CT

144 slope method, was ~100% (data not shown). Canine specific TaqMan gene expression assay
145 for IGF1 (Prod. No. Cf02627846_m1) was purchased from Applied Biosystems (Foster City,
146 CA, USA). Reactions were carried out in an ABI PRISM$_{TM}$ 7500 Sequence Detection System
147 (Applied Biosystems, Darmstadt, Germany) using 96-well optical plates following our
148 protocols (Kowalewski et al., 2006b, 2011). All samples were run in duplicates. Canine
149 GAPDH (GAPDH (forward): 5'-GCT GCC AAA TAT GAC GAC ATC A-3', GAPDH
150 (reverse): 5'-GTA GCC CAG GAT GCC TTT GAG-3', GAPDH TaqMan probe: 5'-TCC
151 CTC CGA TGC CTG CTT CAC TAC CTT-3'; Kowalewski et al., 2006a) and cyclophyllin A
152 (Prod. No. Cf03986523-gH, Applied Biosystems) were used as reference genes. RT-minus
153 controls, *i.e.*, samples without reverse transcription in the reaction were carried out for quality
154 control. No template controls were used in each run substituting cDNA with autoclaved water.
155 Relative gene expression was calculated using the comparative Ct ($\Delta\Delta$Ct) method as reported
156 previously (Kowalewski et al., 2006a and 2011).
157
158 ***Immunohistochemistry***
159 An indirect immunoperoxidase method was applied for protein detection as previously
160 described (Kowalewski et al., 2006a). A rabbit polyclonal antibody for Lep (ARP41697_P050,
161 Aviva Systems Biology, San Diego, USA) and a goat polyclonal antibody recommended for
162 the detection of both short and long LepR isoforms (Ob-R (M-18): sc-1834, Santa Cruz
163 Biotechnology Inc., CA, USA) were applied at a dilution of 1:500 and 1:150, respectively.
164 IGF1 and IGF1R were detected with polyclonal rabbit antibodies purchased from Bioss Inc.
165 (Woburn, MA, USA; bs-0014R diluted 1:300 and bs-0227R diluted 1:350, respectively).
166 Testes sections of 2-3 µm thickness were mounted on SuperFrost Plus microscope slides
167 (Menzel-Gläser, Braunschweig, Germany), deparaffinized in xylol and rehydrated in a graded

ethanol series. Antigen retrieval was induced by heat in 10 mM citrate buffer (pH 6.0) for 15 min at 100°C. Thereafter, slides were placed in 0.3% hydrogen peroxide in methanol to quench endogenous peroxidases and blocked with 1.5% BSA and 10% goat serum (for Lep, IGF1, IGF1R; KPL, Gaithersburg, USA) or 10% horse serum (for LepR; Vector Laboratories, Burlingame, USA). Incubation with the primary antibodies was carried out overnight at +4°C. Biotinylated goat anti-rabbit IgG or horse anti-goat IgG (Vector Laboratories) at a dilution of 1:100 was used as secondary antibody. Signals were enhanced with the avidin/biotinylated peroxidase complex (Vectastain ABC Kit, Vector Laboratories, Burlingame, USA) and color reactions were achieved by applying 3,3'-diaminobenzidine (DAB) as chromogen substrate (Liquid DAB+ substrate Kit, Dako Schweiz AG, Baar, Switzerland). Finally, slides were counterstained with Mayer's hematoxylin, dehydrated in a graded ethanol series and covered with coverslips. Isotype controls were carried out using pre-immune rabbit (for Lep, IGF1, IGF1R) or goat (for LepR) IgG (Vector Laboratories) instead of the primary antibody. The Lep and LepR antibodies applied here were used on canine tissues before (Ressel et al., 2012; Balogh et al., 2015). Additionally, we used mouse liver and skeletal muscle (mouse and dog) as positive control for IGF1 and IGF1R, respectively (data not shown).

Statistical analysis

Normal distribution of data was assessed by the Kolmogorov-Smirnov test. The two-tailed Student's t-test for independent samples was used for the comparison of relative gene expression between adult and prepubertal dogs. Results are presented as mean ± standard deviation of observed data. $P \leq 0.05$ was considered significant. Statistical calculations were carried out with IBM® SPSS® Statistics for Windows, Version 22.0 (Armonk, NY, USA).

Results

Lep and LepR mRNA levels were similar between prepubertal animals and adult dogs (P=0.069 and P=0.37, respectively; Figure 1). Testicular IGF1 and IGF1R gene expression was higher in 8-week-old puppies than in sexually mature dogs (P<0.0001; Figure 1).

In adult animals, strong Lep immunoreactivity was detected in spermatocytes and spermatids, while Leydig cells showed sporadic, weak staining (Figure 2A). In prepubertal animals, intense positive reaction of Lep was present in Leydig and Sertoli cells (Figure 2B). LepR signals in the testes of adult dogs were visible in spermatocytes and spermatids (Figure 2C), but not in Leydig cells. In the puppies, LepR immunoreactivity was present in Leydig cells (Figure 2D). Positive signals were also detected in stromal fibroblasts, which was more abundant in the prepubertal testis. Blood vessel intima and media also stained for Lep and LepR both in adult and prepubertal animals (not shown).

In the testis of sexually mature dogs, IGF1 and IGF1R protein signals were found in spermatogonia within the seminiferous tubules (Figure 3A and 3C, respectively). IGF1 signals in Leydig cells seemed stronger than IGF1R, which showed sporadic, weak staining. In the puppies, Leydig cells as well as gonocytes had positive IGF1 immunostaining, while Sertoli cells had weak signals (Figure 3B). Leydig cells and gonocytes were also positive for IGF1R, and Sertoli cells stained sporadically and weakly (Figure 3D). Blood vessel media had strong immunoreactivity for IGF1R (Figure 3D) and weakly stained for IGF1 in both age groups.

Discussion

Similarly to the findings of El-Hefnawy et al. (2000) in the adult mouse testis, in adult dogs, LepR was expressed in spermatocytes, while Sertoli and Leydig cells were negative. However, we also detected LepR immunostaining in canine spermatids. Localization of Lep was similar

to that of its receptor in adult dogs except that signals were as well present in Leydig cells. This indicates that Lep may have a direct role in canine spermatogenesis. In isolated adult seminiferous tubules in mice, Lep induced signal transducers and activators of transcription (STAT3) signaling, a pathway usually associated with cell differentiation (El-Hefnawy et al., 2000). Based on these findings, the authors concluded that Lep may allow proliferation and renewal of type A spermatogonia and promote differentiation and maturation of spermatocytes to spermatids. We hypothesize similar effects for Lep on spermatocytes and spermatids in the canine testis. Furthermore, this hormone may have anti-apoptotic function in germ cells, as lack of Lep in the ob/ob mice was associated with increased apoptosis rate of spermatocytes (Bhat et al., 2006), although this has not been evaluated in the present study.

Lep's role in steroidogenesis of adult Leydig cells seems to be either negative or not existing according to previous reports in mice and rats (Tena-Sempere et al., 1999 and 2001, Herrid et al., 2008b). The negative role of Lep is supported by findings in humans, where the ratio of LepR positive Leydig cells was negatively correlated with serum T levels (Ishikawa et al., 2007). Our findings in the dog are in contrast with the immunohistochemistry results of the pig testis (Rago et al., 2009), because canine Leydig cells had weak or no expression of Lep and LepR, respectively. It is therefore possible that in the normally functioning adult dog testis Lep has no effect or only minimal modulatory role on T production.

Lep and LepR gene expression was not significantly different between the prepubertal and adult testis. However, in the puppies, Lep staining was intense in Sertoli cells and disappeared in adulthood, which may suggest a specific role for Lep on Sertoli cell development. Furthermore, stronger Lep and LepR protein expression was seen in Leydig cells of prepubertal than in adult dogs. It is possible that Lep stimulates proliferation and

differentiation of these cells in the juvenile testis and may also have a role on T production like it was shown *in vitro* in the immature mouse testis (Herrid et al., 2008b).

IGF1 and IGF1R were co-expressed in spermatogonia and Leydig cells in sexually mature dogs, which is comparable to the localization pattern in stallions (Yoon et al., 2011), while in rats IGF1 was present in spermatocytes (Hansson et al., 1989) and phosphorylated IGF1R in germ cells, Leydig and Sertoli cells (Colón et al., 2007). Similarly to its role in rats, where IGF1 increased DNA synthesis in spermatogonia (Söder et al., 1992) and reduced apoptosis rate in germ cells (Ozkurkcugil et al., 2004), IGF1 may also stimulate spermatogonia proliferation and germ cell survival in dogs, however this was not evaluated here in an *in vitro* assay. The strong immunoreactivity of IGF1 and IGF1R found in canine Leydig cells may indicate a possible action (anti-apoptotic or T metabolism) similarly to that reported in rodents and horses (Manna et al., 2006; Colón et al., 2007; Yoon and Roser, 2011).
According to the findings in rodents, IGF1 is crucial for the development of immature Sertoli cells, as lack of IGF1 and insulin signaling in the murine testis decreased Sertoli cell proliferation during late fetal and early neonatal life resulting in reduced testicular weight and sperm output in adults (Pitetti et al., 2013). It is also needed for proliferation, differentiation and maturation of Leydig cells and for adequate T biosynthesis during postnatal life (Wang et al., 2003; Colón et al., 2007). We found higher mRNA expression of IGF1 and IGF1R in the prepubertal canine testis compared to adults, and based on their protein localization, a stimulatory role in Leydig and Sertoli cell development may be assumed. The presence of both proteins in gonocytes further suggests that the IGF1 system may stimulate germ cell proliferation and differentiation in the juvenile canine testis. Despite significantly higher StAR mRNA levels in the testis of 2-month-old puppies compared to adult dogs, a lower number of

testicular Leydig cells were actually expressing the protein itself (Goericke-Pesch et al., 2013), which would explain the low plasma T levels commonly found during the infantile period in dogs (Mialot et al., 1988). Although in horses, IGF1 did not stimulate T synthesis in immature Leydig cell cultures (Yoon and Roser, 2011), we hypothesize that in prepubertal dogs IGF1 may increase StAR gene expression, but the post-transcriptional processing of the mRNA may not be complete in all Leydig cells and hence T is low.

In conclusion, our descriptive data may suggest that the Lep and IGF systems have distinct roles in canine testicular function, which is also dependent on the developmental stage of the testis. The higher gene expression and/or abundant presence of Lep and IGF1 protein and their receptors in specific cells of the immature testis may be indicative of their roles in Sertoli and Leydig cell proliferation and differentiation or in gonocyte development. In sexually mature animals, Lep could play a role in the development of spermatocytes and spermatids, while IGF1 may be involved in the regulation of spermatogonia proliferation and Leydig cell function.

Acknowledgement

We would like to thank Elisabeth Högger for helping with tissue preparation for IHC.

References

Balogh O, Kowalewski MP, Reichler IM. Leptin and leptin receptor gene expression in the canine corpus luteum during diestrus, pregnancy and after aglepristone-induced luteolysis. Reprod Domest Anim. 2012;47 Suppl 6:40-42.

286 Balogh O, Staub LP, Gram A, Boos A, Kowalewski MP, Reichler IM. Leptin in the canine
287 uterus and placenta: possible implications in pregnancy. Reprod Biol Endocrinol.
288 2015;13:13.

289 Bhat GK, Sea TL, Olatinwo MO, Simorangkir D, Ford GD, Ford BD, Mann DR. Influence of
290 a leptin deficiency on testicular morphology, germ cell apoptosis, and expression levels of
291 apoptosis-related genes in the mouse. J Androl. 2006;27:302-310.

292 Colón E, Zaman F, Axelson M, Larsson O, Carlsson-Skwirut C, Svechnikov KV, Söder O.
293 Insulin-like growth factor-I is an important antiapoptotic factor for rat leydig cells during
294 postnatal development. Endocrinology. 2007;148:128-139.

295 El-Hefnawy T, Ioffe S, Dym M. Expression of the leptin receptor during germ cell
296 development in the mouse testis. Endocrinology. 2000;141:2624-2630.

297 Goericke-Pesch S, Gentil M, Spang A, Kowalewski MP, Failing K, Hoffmann B: Status of the
298 down-regulated canine testis using two different GNRH agonist implants in comparison
299 with the juvenile testis. Reproduction. 2013; 146:517–526.

300 Hansson HA, Billig H, Isgaard J. Insulin-like growth factor I in the developing and mature rat
301 testis: immunohistochemical aspects. Biol Reprod. 1989;40:1321-1328.

302 Herrid M, O'Shea T, McFarlane JR. Ontogeny of leptin and its receptor expression in mouse
303 testis during the postnatal period. Mol Reprod Dev. 2008a;75:874-880.

304 Herrid M, Xia Y, O'Shea T, McFarlane JR. Leptin inhibits basal but not gonadotrophin-
305 stimulated testosterone production in the immature mouse and sheep testis. Reprod Fertil
306 Dev. 2008b;20:519-528.

307 Ishikawa T, Fujioka H, Ishimura T, Takenaka A, Fujisawa M. Expression of leptin and leptin
308 receptor in the testis of fertile and infertile patients. Andrologia. 2007;39:22-27.

309 Kautz E, Gram A, Aslan S, Ay SS, Selçuk M, Kanca H, Koldaş E, Akal E, Karakaş K, Findik
310 M, Boos A, Kowalewski MP. Expression of genes involved in the embryo-maternal
311 interaction in the early-pregnant canine uterus. Reproduction. 2014;147:703-717.

312 Kowalewski MP, Schuler G, Taubert A, Engel E, Hoffmann B. Expression of cyclooxygenase
313 1 and 2 in the canine corpus luteum during diestrus. Theriogenology. 2006a;66:1423-30.

314 Kowalewski MP, Mason JI, Howie AF, Morley SD, Schuler G, Hoffmann B. Characterization
315 of the canine 3β-hydroxysteroid dehydrogenase and its expression in the corpus luteum
316 during diestrus. J Steroid Biochem Mol Biol. 2006b;101:254-62.

317 Kowalewski MP, Beceriklisoy HB, Pfarrer C, Aslan S, Kindahl H, Kücükaslan I, Hoffmann B.
318 Canine placenta: a source of prepartal prostaglandins during normal and antiprogestin-
319 induced parturition. Reproduction. 2010;139:655-64.

320 Kowalewski MP, Meyer A, Hoffmann B, Aslan S, Boos A. Expression and functional
321 implications of peroxisome proliferator-activated receptor gamma (PPARγ) in canine
322 reproductive tissues during normal pregnancy and parturition and at antiprogestin induced
323 abortion. Theriogenology. 2011;75:877-86.

324 Manna PR, Chandrala SP, King SR, Jo Y, Counis R, Huhtaniemi IT, Stocco DM. Molecular
325 mechanisms of insulin-like growth factor-I mediated regulation of the steroidogenic acute
326 regulatory protein in mouse leydig cells. Mol Endocrinol. 2006;20:362-378.

327 Mialot JP, Thibier M, Toublanc JE, Castanier M, Scholler R. Plasma concentration of
328 luteinizing hormone, testosterone, dehydroepiandrosterone, androstenedione between birth
329 and one year in the male dog: longitudinal study and hCG stimulation. Andrologia.
330 1988;20:145-154.

Ozkurkcugil C, Yardimoglu M, Dalcik H, Erdogan S, Gokalp A. Effect of insulin-like growth factor-1 on apoptosis of rat testicular germ cells induced by testicular torsion. BJU Int. 2004;93:1094-1097.

Peters MAJ, Mol JA, van Wolferen ME, Oosterlaken-Dijksterhuis MA, Teerds KJ, van Sluijs FJ. Expression of the insulin-like growth factor (IGF) system and steroidogenic enzymes in canine testis tumors. Reprod Biol Endocrinol. 2003;1:22.

Pitetti JL, Calvel P, Zimmermann C, Conne B, Papaioannou MD, Aubry F, Cederroth CR, Urner F, Fumel B, Crausaz M, Docquier M, Herrera PL, Pralong F, Germond M, Guillou F, Jégou B, Nef S. An essential role for insulin and IGF1 receptors in regulating Sertoli cell proliferation, testis size, and FSH action in mice. Mol Endocrinol. 2013:27:814–827.

Rago V, Aquila S, Guido C, Carpino A. Leptin and its receptor are expressed in the testis and in the epididymis of young and adult pigs. Anat Rec (Hoboken). 2009;292:736-745.

Ressel L, Finotello R, Innocenti VM, Vannozzi I, Poli A. Preliminary report on the expression of leptin and leptin receptor (ObR) in normal, hyperplastic and neoplastic canine mammary tissues. Res Vet Sci. 2012;93:343-349.

Söder O, Bang P, Wahab A, Parvinen M. Insulin-like growth factors selectively stimulate spermatogonial, but not meiotic, deoxyribonucleic acid synthesis during rat spermatogenesis. Endocrinology. 1992;131:2344-2350.

Tena-Sempere M, Pinilla L, González LC, Diéguez C, Casanueva FF, Aguilar E. Leptin inhibits testosterone secretion from adult rat testis in vitro. J Endocrinol. 1999;161:211-218.

Tena-Sempere M, Manna PR, Zhang FP, Pinilla L, González LC, Diéguez C, Huhtaniemi I, Aguilar E. Molecular mechanisms of leptin action in adult rat testis: potential targets for

353 leptin-induced inhibition of steroidogenesis and pattern of leptin receptor messenger
354 ribonucleic acid expression. J Endocrinol. 2001;170:413-423.

355 Yoon MJ, Roser JF. A synergistic effect of insulin-like growth factor (IGF-I) on equine
356 luteinizing hormone (eLH)-induced testosterone production from cultured Leydig cells of
357 horses. Anim Reprod Sci. 2011;126:195-199.

358 Yoon MJ, Berger T, Roser JF. Localization of insulin-like growth factor-I (IGF-I) and IGF-I
359 receptor (IGF-IR) in equine testes. Reprod Dom Anim. 2011;46:221–228.

360 Wang GM, O'Shaughnessy PJ, Chubb C, Robaire B, Hardy MP. Effects of insulin-like growth
361 factor I on steroidogenic enzyme expression levels in mouse leydig cells. Endocrinology.
362 2003;144:5058-5064.

363 **Figure captions**

364 Figure 1

365 Relative gene expression of leptin (Lep), leptin receptor (LepR), insulin-like growth factor 1
366 (IGF1) and its receptor (IGF1R) in the testis of healthy adult and prepubertal (8-week-old)
367 dogs. Asterix above the respective column denotes significant difference between the groups.

368

369 Figure 2

370 Lep protein distribution in the adult (A) and prepubertal (B) testis, and LepR protein
371 localization in the adult (C) and prepubertal testis (D) is shown. A: Positive Lep
372 immunoreactivity is present in spermatocytes and spermatids (black arrows). Leydig cells
373 show sporadic, weak staining (lower right inset). B: In prepubertal dogs, both Leydig cells
374 (white arrowheads) and Sertoli cells (black arrowheads) stain positive. Gonocytes did not stain
375 (black arrows). C and D: In the adult testis, spermatocytes and elongated spermatids show
376 positive LepR immunoreactivity (C: black arrows). LepR protein is also detected in round and
377 elongated spermatids (D: black arrows). E: LepR in the prepubertal testis is visible in Leydig
378 cells (white arrowheads), while Sertoli cells (black arrowheads) and gonocytes (black arrow)
379 did not stain.
380 Insets showing isotype controls for Lep or LepR are in the right upper corner of the respective
381 image. Scale bar: 25 µM

382

383 Figure 3

384 IGF1 protein distribution in the adult (A) and prepubertal (B) testis, and IGF1R protein
385 localization in the adult (C) and prepubertal testis (D) is shown. A: Positive IGF1 signals are
386 present in spermatogonia (black arrows), and intense staining is also present in Leydig cells

(white arrowheads) of adult dogs. B: Leydig cells (white arrowheads) and gonocytes (black arrows) show positive IGF1 immunoreactivity in the prepubertal testis, and Sertoli cells stain weakly (black arrowheads). C: In the testis of adult dogs, strong positive IGF1R signals are present in spermatogonia (black arrows), while staining is weaker in Leydig cells (white arrowheads). D: Signals for IGF1R are present in gonocytes (black arrows), Leydig cells (white arrowheads), and sporadically and weakly in Sertoli cells (black arrowheads) of prepubertal animals. Note IGF1R immunoreactivity in blood vessels (white arrows). Insets show the isotype controls for IGF1 or IGF1R in the respective image. Scale bar: 25 µM